II. The Office Action, and Responses Thereto

The Action rejects the pending claims on several grounds. These grounds are individually addressed below.

A. Rejection of Claims 1-14 For Alleged Lack of Written Description

The Action rejects claims 1-14 under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the written description requirement. The Action asserts that the claims contain subject matter that was not described in the specification so as to reasonably convey to the practitioner that, at the time the invention was made, the applicant had possession of the claimed invention. According to the Action, the claims are

> drawn to a plant comprising an expression cassette comprising a nucleic acid sequence encoding a farnesyltransferase inhibitor of unspecified structure and function, including an unspecified protein inhibitor, and to a method of inhibiting farnesyltransferase in a plant by transforming a plant with said expression cassette . . .

Action, at page 3.

The Action cites *Regents of the University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997) as indicating that a "description of a genus of cDNAs may be achieved by means of recitation of a representative number of cDNAs, defined by nucleotide sequence, falling with the scope of the genus or of a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus." Action at page 4, quoting *Lilly*, supra. The Action states that the Applicants have "not described a representative number of species falling within the scope of the claimed genus, nor the structural features unique to the genus. Applicants traverse.

The Action correctly states that the purpose of the written description requirement is to show that the Applicants had possession of the invention at the time of filing. Contrary to the suggestion of the Action, however, Applicants had possession of the invention as of the date of filing.

At the September 29, 1999, filing of the international application from which the present application claims priority, literally scores of peptide and peptidomimetic inhibitors of farnesyltransferase (sometimes hereafter referred to as "farnesyl protein transferase" or as "FTase") were known in the art. Indeed, two of the references cited by the Action *against* the claims, Reiss et al., Cell 62:81-88 (1990) (hereafter, "Reiss") and Tamanoi, TIBS 18:349-353 (1993) (hereafter, "Tamanoi"), show that as early as 1990, the basic CAAX motif for competitive inhibitors of the enzyme was already known and teach a number of inhibitors of FTase.

Turning to the guidance provided by the specification, the specification recites no fewer than 10 patents that issued on novel farnesyltransferase inhibitors between just 1998 and the filing of the priority international application in September 1999. Specification, at page 15, lines 8-9. These patents were incorporated by reference into the specification (see, page 33, lines 20-21) and therefore are and legally always have been part of the specification's teachings.

As stated by U.S. Patent No. 5,914,341, one of the patents incorporated by reference:

> Inhibitors of farnesyl-protein transferase (FPTase) have been described in two general classes. The first are analogs of farnesyl diphosphate (FPP), while the second class of inhibitors is related to the protein substrates (e.g., Ras) for the enzyme. The peptide derived inhibitors that have been described are generally cysteine containing molecules that are related to the CAAX motif that is the signal for protein prenylation. (Schaber et al., ibid; Reiss et. al., ibid; Reiss et al., PNAS, 88:732-736 (1991)). Such inhibitors may inhibit protein prenylation while serving as alternate substrates for the farnesyl-protein transferase enzyme, or may be purely competitive inhibitors (U.S. Pat. No. 5,141,851, University of Texas; N. E. Kohl et al., Science, 260:1934-1937 (1993); Graham, et al., J. Med. Chem., 37, 725 (1994)).

'341 patent. at column 2, lines 20-33. (A copy of the cited page of the '341 patent is enclosed for the Examiner's convenience.) Similarly, another of the incorporated patents, U.S. Patent No. 5,856,310, states:

Potential inhibitor candidates are CA_1A_2X tetrapeptides which have been shown to be farnesylated by p21ras farnesyltransferase and appear to be potent inhibitors of this enzyme in vitro [citations to six references omitted]. Competition studies have demonstrated that CA_1A_2X peptides with the greatest inhibitory activity are those where A_1 and A_2 are hydrophobic peptides with charged or hydrophilic residues in the central positions demonstrating very little inhibitory activity (18,21,23).

The research efforts directed towards farnesyltransferase and the inhibition of its activity are further illustrated by the following patents or published patent applications:

U.S. Pat. No. 5,141,851

WO 91/16340

WO 92/18465

EPA 0456180 A1

EPA 0461869 A2

EPA 0512865 A2

EPA 0520823 A2

EPA 0523873 A1

. . .

EPA 0461869 describes compounds which inhibit farnesylation of Ras protein of the formula:(SEQ ID NO: 1)

Cys-Aaa^1-Aaa^2-Xaa

where Aaa^1 and Aaa^2 are aliphatic amino acids and Xaa is an amino acid. The aliphatic amino acids which are disclosed are Ala, Val, Leu and Ile. Preferred compounds are those where Aaa^1 is Val, Aaa^2 is Leu, Ile or Val and Xaa is Ser or Met. Preferred specific compounds are:

Cys-Val-Leu-Ser

Cys-Val-Ile-Met

Cys-Val-Val-Met

> U.S. Pat. Nos. 5,141,851 and WO 91/16340 disclose the purified farnesyl protein transferase and certain peptide inhibitors therefor, including, for example, CVIM, TKCVIM and KKSKTKCVIM (SEQ ID NO:2 through SEQ ID NO:4),
>
> WO 92/18465 discloses certain farnesyl compounds which inhibit the enzymatic methylation of proteins including ras proteins.

'310 patent. at column 1, line 44, to column 2, line 50. (A copy of the cited page of the '310 patent is enclosed for the Examiner's convenience.)

Thus, both the '341 and the '310 patents contain teachings about the structure of peptides that can be used to inhibit farnesyltrasferase activity. Further, U.S. Pat. No. 5,141,851 (the "'851 patent"), which is cited by both the '341 and 310 patents in the portions quoted above, states:

> In preferred embodiments, the farnesyl transferase inhibitor of the present invention will include a farnesyl acceptor or inhibitory amino acid sequence having the amino acids --C--A--A--X, wherein: C=cysteine; A=any aliphatic, aromatic or hydroxy amino acid; and X=any amino acid.
>
> Typically, the farnesyl acceptor or inhibitory amino acid sequence will be positioned at the carboxy terminus of the protein or peptide such that the cysteine residue is in the fourth position from the carboxy terminus.
>
> In preferred embodiments, the inhibitor will be a relatively short peptide such as a peptide from about 4 to about 10 amino acids in length. To date, the most preferred inhibitor tested is a tetrapeptide which incorporates the --C1'A--A--X recognition structure. It is possible that even shorter peptides will ultimately be preferred for practice of the invention in that the shorter the peptide, the greater the uptake by such peptide by biological systems, and the reduced likelihood that such a peptide will be destroyed or otherwise rendered biologically ineffective prior to effecting inhibition. However, numerous suitable inhibitory peptides have been prepared and tested by the present inventors, and shown to inhibit enzymatic activities virtually completely, at reasonable concentrations,

> e.g., between about 1 and 3 μM (with 50% inhibitions on the order of 0.1 to 0.5 μM).
>
> . . .
>
> Exemplary peptides which have been prepared, t[]ested and shown to inhibit farnesyl transferase at an IC_{50} of between 0.01 and 10 μM include CVIM; KKSKTKCVIM; TKCVIM; RASNRSCAIM; TQSPQNCSIM; CIIM; CVVM; CVLS; CVLM; CAIM; CSIM; CCVQ; CIIC; CIIS; CVIS; CVLS; CVIA; CVIL; CLIL; CLLL; CTVA; CVAM; CKIM; CLIM; CVLM; CFIM; CVFM; CVIF; CEIM; CGIM; CPIM; CVYM; CVTM; CVPM; CVSM; CVIF; CVIV; CVIP; CVII.

'851 patent at column 8, lines 32-41. (For the Examiner's convenience, the relevant page of the '851 patent is attached.)

Thus, the '851 patent discloses no fewer than 39 peptides that were tested and found to inhibit farnesyltransferase activity. The '851 patent issued in 1992, and therefore was available to the practitioner for years before the priority date of the present application. Persons of skill in this art, who typically hold Ph.D.s, were clearly enabled to design nucleic acids that encoded these various peptides. Similarly, not only are numerous promoters known in the art for expressing nucleic acids in plants, see, e.g., specification at page 24, lines 19-32, but the art also taught promoters for expressing nucleic acids specifically in guard cells. See, e.g., page 15, lines 21-29.

As MPEP §2164.01 reminds the Examining Corps, "[a] patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987)." In the case of the present application, there was no need for the specification to teach the practitioner nucleic acids that would encode inhibitors of farnesyltransferase activity when such inhibitors had already been known for years.

Lilly does not apply to situations like that presented by the present application. In *Lilly*, the patent holder had taught the cDNA for a rat protein, and was seeking a holding of infringement of a human cDNA sequence the application claimed but did not teach. The Court

had previously noted in earlier cases that, due to the degeneracy of the genetic code, a multitude of nucleic acids sequences could encode any given protein. Thus, while the amino acid sequence of the human protein was known, it did not give the patentee a basis for claiming a particular nucleic acid sequence as the human gene sequence given the multitude of possibilities of nucleic acid sequence that could have encoded it. It is in this context that the Court indicated that the patentee had not shown possession of the claimed invention at the time of filing.

In the present case, however, dozens of inhibitors of farnesyltransferase were known in the art for years before the filing date, as were promoters permitting expression of those inhibitors in plants. Further, and in contrast to the situation in *Lilly*, the claims are not drawn to any particular gene or a preferred nucleic acid sequence encoding, for example, the Cys-Val-Leu-Ser peptide sequence or the other peptides set forth in the '310 and '851 patents. Any of the multitude of sequences that could encode the peptides would be satisfactory and would be within the scope of the claim.

The Applicants, like others in the art, had possession of a large number of inhibitors of FTase at the time of filing. Given the Federal Circuit's dictates that the specification does not need to set forth – and preferably omits – information that would be known to persons of skill in the art, it was unnecessary to recite specific sequences in the specification merely to show that they also had a copy of the genetic code and could convert the peptide sequences into corresponding nucleic sequences. The Applicants clearly had possession of the invention at the time of filing.

Reconsideration of the rejection in light of the above comments is respectfully requested.

B. Rejection of Claims 1-14 For Alleged Lack of Enablement

Claims 1-14 are rejected under 35 U.S.C. § 112, first paragraph as allegedly not enabled. Action, at page 4. The Action contends that the invention is not enabled

> because the specification does not provide guidance with respect to the nature and identity of the farnesyltransferase inhibitors that would be encoded by the nucleic acid sequence of the expression cassette, or how to

> express the inhibitors in a manner that would confer a useful phenotype on a plant transformed therewith. Such guidance is necessary because the effect of expressing a nucleic acid encoding a farnesyltransferase inhibitor in a plant is unpredictable.

Action, at page 5. The Action contends that the alleged unpredictability is present because, (a) expression methods must be specifically adapted to achieve a desired phenotype, (b) because farnesyltransferase inhibition is dependent on inhibitor concentration and will vary between different types of inhibitors, and (c) because compounds that inhibit farnesyltransferase in vitro may be unstable in vivo. Applicants traverse. For ease of reference, each of the grounds of rejection are set forth and responded to individually below.

(1) Contention that the expression methods must be specifically adopted in order to achieve a particular desired phenotype

The Action first contends that the expression methods must be specifically adopted in order to achieve a particular desired phenotype, as different levels of protein expression produce different phenotypes. Action, at pages 5-6, bridging paragraph.

As an initial matter, Applicants respectfully point out that the Office "has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention." MPEP § 2164.04. The MPEP further reminds the examining corps that, under the court's decision in *In re Marzocchi*, 169 USPQ 367, 370 (CCPA 1971), the Patent Office has the burden to state not only "why it doubts the truth or accuracy of any statement in a supporting disclosure," but also "to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." MPEP § 2164.04, quoting *In re Marzocchi, supra*, at page 370 (emphasis in original).

Applicants respectfully submit that the Action does not meet this burden. The Action cites no cases or art showing that there is any unpredictability in the activity of farnesyltransferase inhibitors generally or specifically as applied to the case of inhibiting

farnesyltransferase in plants. Further, the Action points to no particular reason that expression of farnesyltransferase inhibitors would require more than routine experimentation to adapt standard methods to permit expression of the inhibitors.

The Action appears to assume that, if any experimentation is required, the invention is not enabled. This is not correct. In fact, an invention can require considerable experimentation to practice so long as that experimentation is not "undue." Whether or not experimentation is "undue" is determined under criteria that were articulated by the Federal Circuit Court of Appeals in *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

The *Wands* criteria are: (1) the breadth of the claims; (2) the nature of the invention; (3) the state of the prior art; (4) the level of one of ordinary skill; (5) the level of predictability in the art; (6) the amount of direction provided by the inventor; (7) the existence of working examples; and (8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

The Action does not even acknowledge the *Wands* factors, let alone indicate that there is any reason that the application does not satisfy them. It is clear that the Action has failed to apply the correct standard to determine whether any experimentation to practice the invention would be "undue." The Action is therefore grounded on an incorrect basis and should be withdrawn on this basis alone.

Further, Applicants respectfully maintain that application of the *Wands* criteria to the present claims shows that persons of skill in the art were fully enabled to use the invention as claimed without undue experimentation:

Factor 1: The claims are narrowly drawn to plants expressing recombinant expression cassettes encoding inhibitors of a specific enzyme and to methods of inhibiting that enzyme in plants by introducing such recombinant expression cassettes.

Factor 2: The invention is related to the surprising discovery that inhibition of farnesyltransferase activity in plants results in increasing their resistance to drought.

Factor 3: The state of the prior art sets forth significant teachings on inhibitors of farnesyltransferase. For example, the '851 patent cited above teaches no fewer than 39 peptides each of which was tested and found to inhibit farnesyltransferase activity at micromolar

concentrations. Further, numerous promoters were known in the art for driving expression of nucleic acids in plants generally (*see, e.g.*, specification at page 24, lines 19-32) and in guard cells specifically (*see, e.g.*, page 15, lines 24-29).

Factor 4: Persons of skill in this art are typically Ph.D. plant scientist. Therefore, the level of skill in the art is very high, and the amount of guidance that needs to be provided is correspondingly limited.

Factor 5: Substantial predictability can be found in the relevant field. As noted in the specification, techniques for transforming plants by various techniques were known and used for some 15 years prior to the priority date of the application. See, specification at page 25, line

Factor 6: The specification provides ample guidance as to how to make and use the invention as claimed. For example, the specification teaches the preparation of recombinant vectors (see, page 24, lines 10-18), suitable promoters (page 24, lines 19-32), and production of transgenic plants (see, page 25, line 23 to page 26, line 27). Further, the specification contains an example of the production of fusion constructs and of production of transgenic plants, using the well-known GUS reporter gene. See, specification at page 32, lines 18-28.

Factor 7: As noted in the preceding section, the specification sets forth a working example showing the expression of a transgene in *Arabidopsis* guard cells. See, specification at page 32, lines 18-28. While the example relates to the expression of a reporter gene, the Action sets forth no reasoning or art showing that there would be any different result if the sequence encoding the reporter were to be replaced instead with a sequence encoding instead a farnesyltransferase inhibitor, such as one of the 39 peptides described in the '851 patent.

Factor 8: Lastly, the amount of experimentation necessary for an artisan to make or use the invention as claimed is modest. As noted in the previous paragraph, techniques and methodologies for expressing farnesyltransferase inhibitors are set forth in the specification. Thus, the specification sets forth all the information an artisan needs to express any particular farnesyltransferase inhibitor in any particular plant of interest.

In summation, the rejection of the claims as not enabled on the first of the grounds articulated (a) fails to carry the burden on the Office imposed by *Marzocchi* and MPEP §2164.04

to set forth reasoning or evidence to counter the specification's presentation regarding the use of endosome pH raising agents and (2) fails to consider the correct criteria for determining whether any experimentation that might be necessary would be "undue." Correctly considered, the claims are fully enabled and any experimentation that might be necessary is not undue. The rejection set forth in the Action should be reconsidered and, upon reconsideration, withdrawn.

(2) Contention that there is unpredictability because different inhibitors will differ in the concentration required to inhibit farnesyltransferase.

The Action next contends that different farnesyltransferase inhibitors differ in the concentrations required to inhibit enzyme activity. The Action cites the Tamanoi reference as teaching that the IC_{50}s of various farnesyltransferase inhibitors varies from 0.01 μM to 21 μM among 10 different types of farnesyltransferase inhibitors. Further, the Action cites the Reiss reference as teaching that different species of peptide inhibitors vary in the concentration at which they inhibit farnesyltransferase activity.

As with the contention discussed in the preceding section, the Action appears to assume that, if there is any variability, and any experimentation is required, the invention is not enabled. This is not correct. As previously pointed out, an invention can require considerable experimentation to practice so long as that experimentation is not "undue," and whether or not any experimentation is or is not undue is determined under the criteria set forth in *Wands*. As also previously noted, the Action does not even acknowledge the *Wands* factors, let alone indicate that there is any reason that the application does not satisfy them. It is thus again clear that the Action has not applied the correct standard to determine whether any experimentation to practice the invention would be "undue."

Applicants observe that the Action has set forth neither reasoning nor art indicating that producing FTase inhibitors in plants would require any more experimentation than producing plants that express, for example, insect toxins from the bacterium *Bacillus thurengiensis*. Yet, plants expressing such toxins are commercially available. It is indeed routine in the art to generate a number of transgenic plants, to select for those expressing the desired phenotype, and to bulk up seed production of plants with the desired phenotype. The

Action has not alleged or shown otherwise, or shown why such experimentation would be undue under the *Wands* standards.

Applicants request reconsideration, and withdrawal, of the rejection in light of the remarks above.

(3) Contention that there is unpredictability because compounds that inhibit farnesyltransferase in vitro may be unstable in vivo.

Finally, the Action contends that there is unpredictability because compounds that inhibit FTase activity in vitro may be unstable in vivo. In this regard, the Action cites Lerner et al. Anticancer Drug Res 12(4):229-238 (1997) ("Lerner"), and particularly pages 231-232, as stating that CAAX tetrapeptides had to be chemically modified to increase their stability in vivo. Action, at page 6.

Applicants respectfully observe that this ground of alleged unpredictability is simply inapplicable to the claims under examination. With reference to CAAX tetrapeptide FTase inhibitors, Lerner states at the place emphasized by the Action:

> FTase farnesylates simple CAAX tetrapeptides as efficiently as it farnesylates the full-length Ras proteins, and these tetrapeptides are potent competitive inhibitors of the enzyme in vitro (IC50 = 20-200 nM) (Reiss et al., 1990). These crucial observations [led] several research groups to target the CAAX tetrapeptide as a novel anti-cancer drug development strategy. Major efforts in this area have focused on improving the stability of the tetrapeptides towards proteolytic degradation, increasing their cellular uptake and increasing the selectivity of inhibition of FTase over the closely related GGTase I."

Lerner, at page 231 (emphasis added.

The portion quoted, regarding increasing the stability of the tetrapeptides towards proteolytic degradation and then increasing their cellular uptake, shows that Lerner is referring to the exposure of these tetrapeptides to proteases in human serum, that is, in the extracellular fluids, before the peptides are taken up by the target cells. The FTase inhibitors recited in the

claims, however, are expressed within the cells expressing them; they are therefore not exposed to extracellular fluids. The Action shows no nexus between Lerner's concerns about the stability of tetrapeptides administered into the circulation as anti-cancer agents and the expression of the same peptides within plant cells to inhibit stomatal opening. Even assuming that plant tissues have endogenous proteases in extracellular fluids analogous to the proteases present in human serum -- which the Action neither alleges nor shows -- it would not constitute a showing that stability is an issue with regard to inhibitors expressed and acting within a cell.

Accordingly, this ground of alleged unpredictability is simply not applicable to the claims under rejection. Reconsideration of the rejection in light of the above remarks is respectfully requested.

(4) Summary

In short, none of the articulated grounds for alleged lack of enablement withstands scrutiny. Reconsideration and withdrawal of the rejection is respectfully requested.

III. Filing Receipt

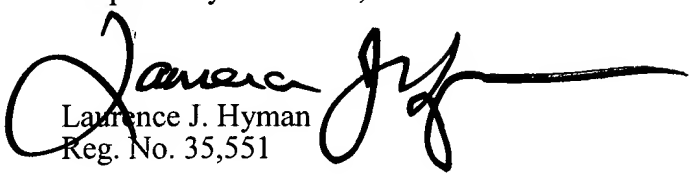
As noted in the Status Request Letter dated September 16, 2003, Applicants have never received a filing receipt for the captioned application. Applicants respectfully request the Examiner's assistance in seeing that a filing receipt is provided for the application.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, she is invited to telephone the undersigned at 415-576-0200.

Respectfully submitted,



Laurence J. Hyman
Reg. No. 35,551

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 415-576-0200
Fax: 415-576-0300
Attachments
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INHIBITORS OF FARNESYL-PROTEIN TRANSFERASE

RELATED APPLICATION INFORMATION

This application is a continuation of now abandoned U.S. Ser. No. 08/786,520, filed on Jan. 21, 1997, which claimed domestic priority from U.S. Provisional Application, U.S. Ser. No. 60/010,799, filed on Jan. 30, 1996, which is now abandoned.

BACKGROUND OF THE INVENTION

The Ras proteins (Ha-Ras, Ki4a-Ras, Ki4b-Ras and N-Ras) are part of a signalling pathway that links cell surface growth factor receptors to nuclear signals initiating cellular proliferation. Biological and biochemical studies of Ras action indicate that Ras functions like a G-regulatory protein. In the inactive state, Ras is bound to GDP. Upon growth factor receptor activation Ras is induced to exchange GDP for GTP and undergoes a conformational change. The GTP-bound form of Ras propagates the growth stimulatory signal until the signal is terminated by the intrinsic GTPase activity of Ras, which returns the protein to its inactive GDP bound form (D. R. Lowy and D. M. Willumsen, *Ann. Rev. Biochem.* 62:851–891 (1993)). Mutated ras genes (Ha-ras, Ki4a-ras, Ki4b-ras and N-ras) are found in many human cancers, including colorectal carcinoma, exocrine pancreatic carcinoma, and myeloid leukemias. The protein products of these genes are defective in their GTPase activity and constitutively transmit a growth stimulatory signal.

Ras must be localized to the plasma membrane for both normal and oncogenic functions. At least 3 post-translational modifications are involved with Ras membrane localization, and all 3 modifications occur at the C-terminus of Ras. The Ras C-terminus contains a sequence motif termed a "CAAX" or "Cys-Aaa^1-Aaa^2-Xaa" box (Cys is cysteine, Aaa is an aliphatic amino acid, the Xaa is any amino acid) (Willumsen et al., *Nature* 310:583–586 (1984)). Depending on the specific sequence, this motif serves as a signal sequence for the enzymes farnesyl-protein transferase or geranylgeranyl-protein transferase, which catalyze the alkylation of the cysteine residue of the CAAX motif with a C_{15} or C_{20} isoprenoid, respectively. (S. Clarke., *Ann. Rev. Biochem.* 61:355–386 (1992); W. R. Schafer and J. Rine, *Ann. Rev. Genetics* 30:209–237 (1992)). The Ras protein is one of several proteins that are known to undergo post-translational farnesylation. Other farnesylated proteins include the Ras-related GTP-binding proteins such as Rho, fungal mating factors, the nuclear lamins, and the gamma subunit of transducin. James, et al., *J. Biol. Chem.* 269, 14182 (1994) have identified a peroxisome associated protein Pxf which is also farnesylated. James, et al., have also suggested that there are farnesylated proteins of unknown structure and function in addition to those listed above.

Inhibition of farnesyl-protein transferase has been shown to block the growth of Ras-transformed cells in soft agar and to modify other aspects of their transformed phenotype. It has also been demonstrated that certain inhibitors of farnesyl-protein transferase selectively block the processing of the Ras oncoprotein intracellularly (N. E. Kohl et al., *Science*, 260:1934–1937 (1993) and G. L. James et al., *Science*, 260:1937–1942 (1993). Recently, it has been shown that an inhibitor of farnesyl-protein transferase blocks the growth of ras-dependent tumors in nude mice (N. E. Kohl et al., *Proc. Natl. Acad. Sci U.S.A.*, 91:9141–9145 (1994) and induces regression of mammary and salivary carcinomas in ras transgenic mice (N. E. Kohl et al., *Nature Medicine*, 1:792–797 (1995).

Indirect inhibition of farnesyl-protein transferase in vivo has been demonstrated with lovastatin (Merck & Co., Rahway, N.J.) and compactin (Hancock et al., ibid; Casey et al., ibid; Schafer et al., *Science* 245:379 (1989)). These drugs inhibit HMG-CoA reductase, the rate limiting enzyme for the production of polyisoprenoids including farnesyl pyrophosphate. Farnesyl-protein transferase utilizes farnesyl pyrophosphate to covalently modify the Cys thiol group of the Ras CAAX box with a farnesyl group (Reiss et al., Cell, 62:81–88 (1990); Schaber et al., *J. Biol. Chem.*, 265:14701–14704 (1990); Schafer et al., *Science*, 249:1133–1139 (1990); Manne et al., *Proc. Natl. Acad. Sci USA*, 87:7541–7545 (1990)). Inhibition of farnesyl pyrophosphate biosynthesis by inhibiting HMG-CoA reductase blocks Ras membrane localization in cultured cells. However, direct inhibition of farnesyl-protein transferase would be more specific and attended by fewer side effects than would occur with the required dose of a general inhibitor of isoprene biosynthesis.

Inhibitors of farnesyl-protein transferase (FPTase) have been described in two general classes. The first are analogs of farnesyl diphosphate (FPP), while the second class of inhibitors is related to the protein substrates (e.g., Ras) for the enzyme. The peptide derived inhibitors that have been described are generally cysteine containing molecules that are related to the CAAX motif that is the signal for protein prenylation. (Schaber et al., ibid; Reiss et. al., ibid; Reiss et al., *PNAS*, 88:732–736 (1991)). Such inhibitors may inhibit protein prenylation while serving as alternate substrates for the farnesyl-protein transferase enzyme, or may be purely competitive inhibitors (U.S. Pat. No. 5,141,851, University of Texas; N. E. Kohl et al., *Science*, 260:1934–1937 (1993); Graham, et al., *J. Med. Chem.*, 37, 725 (1994)). In general, deletion of the thiol from a CAAX derivative has been shown to dramatically reduce the inhibitory potency of the compound. However, the thiol group potentially places limitations on the therapeutic application of FPTase inhibitors with respect to pharmacokinetics, pharmacodynamics and toxicity. Therefore, a functional replacement for the thiol is desirable.

It has recently been reported that farnesyl-protein transferase inhibitors are inhibitors of proliferation of vascular smooth muscle cells and are therefore useful in the prevention and thereapy of arteriosclerosis and diabetic disturbance of blood vessels (JP H7-112930).

It has recently been disclosed that certain tricyclic compounds which optionally incorporate a piperidine moiety are inhibitors of FPTase (WO 95/10514, WO 95/10515 and WO 95/10516). Imidazole-containing inhibitors of farnesyl protein transferase have also been disclosed (WO 95/09001 and EP 0 675 112 A1).

It is, therefore, an object of this invention to develop peptidomimetic compounds that do not have a thiol moiety, and that will inhibit farnesyl-protein transferase and thus, the post-translational farnesylation of proteins. It is a further object of this invention to develop chemotherapeutic compositions containing the compounds of this invention and methods for producing the compounds of this invention.

SUMMARY OF THE INVENTION

The present invention comprises small molecule peptidomimetic carbamate-containing compounds which inhibit the farnesyl-protein transferase. The instant compounds lack a thiol moiety and thus offer unique advantages in terms of improved pharmacokinetic behavior in animals, prevention of thiol-dependent chemical reactions, such as rapid autoxi-

INHIBITION OF FARNESYLTRANSFERASE

This is a continuation division of application Ser. No. 08/062,287, filed May 18, 1993.

INTRODUCTION

The present invention relates to novel peptidomimetics and other compounds which are useful as inhibitors of p21ras farnesyltransferase. Farnesylation is required for the cancer causing activity of the oncogene product p21ras. Hence there is considerable interest in inhibiting farnesylation.

The invention was supported by grants from the American Cancer Society and the National Cancer Institute (NIH).

BACKGROUND OF THE INVENTION

Ras oncogenes are the most frequently identified activated oncogenes in human tumors (1–3). It is known that the ras oncogenes encode 21,000 dalton G-proteins (p21ras) which play an essential role in growth factor signal transduction, proliferation and malignant transformation (1–7). Association of p21ras with the plasma membrane is required for its transforming activity (8,9). Post-translational events leading to membrane association of p21ras have previously been disclosed (10–14). The p21ras proteins are first made as pro-p21ras in the cytosol where they are modified on cysteine 186 of their carboxyl terminal sequence CA_1A_2X (C=cysteine, A_1 and A_2=isoleucine, leucine or valine and X=methionine or serine) by the cholesterol biosynthesis intermediate farnesyl pyrophosphate (FPP). This farnesylation reaction is then followed by peptidase removal of the A_1A_2X tripeptide and carboxymethylation of the remaining cysteine. The processed p21ras proteins associate with the inner surface of the plasma membrane and are further modified on cysteines 181–184 by another lipid, palmitic acid (10–14).

p21Ras farnesyltransferase, the enzyme responsible for catalyzing the transfer of farnesyl, a 15-carbon isoprenoid, from FPP to the cysteine of the CA_1A_2X carboxyl terminus of p21ras, has been purified to homogeneity from rat brain (15,16). The enzyme is a heterodimer composed of α and β subunits of molecular weights 49 and 46 kDa, respectively (17). The β subunit has been shown (17) to bind p21ras and the α subunit is believed to bind FPP. Because p21ras farnesylation and subsequent membrane association are required for p21ras transforming activity, it has been proposed that p21ras farnesyltransferase would be a useful anticancer therapy target. Accordingly, an intensive search for inhibitors of the enzyme is underway (18–24). Potential inhibitor candidates are CA_1A_2X tetrapeptides which have been shown to be farnesylated by p21ras farnesyltransferase and appear to be potent inhibitors of this enzyme in vitro (15,18,21–24). Competition studies have demonstrated that CA_1A_2X peptides with the greatest inhibitory activity are those where A_1 and A_2 are hydrophobic peptides with charged or hydrophilic residues in the central positions demonstrating very little inhibitory activity (18,21,23).

The research efforts directed towards farnesyltransferase and the inhibition of its activity are further illustrated by the following patents or published patent applications:

U.S. Pat. No. 5,141,851

WO 91/16340

WO 92/18465

EPA 0456180 A1

EPA 0461869 A2

EPA 0512865 A2

EPA 0520823 A2

EPA 0523873 A1

Of the above disclosures, EPA 0520823 A2 discloses compounds which are useful in the inhibition of farnesyl-

protein transferase and the farnesylation of the oncogene protein ras. The compounds of EPA 0520823 A2 are illustrated by the formula:

Cys-Xaa^1-Xaa^2-Xaa^3

or pharmaceutically acceptable salts thereof, wherein Cys is a cysteine amino acid;

Xaa^{1}is an amino acid in natural L-isomer form;

dXaa^2 is an amino acid in unnatural D-isomer form; and

Xaa^3 is an amino acid in natural L-isomer form.

The preferred compounds are said to be CV(DI)S and CV(DI)M, the amino acids being identified by conventional 3 letter and single letter abbreviations as follows:

Cysteine	Cys	C
Glycine	Gly	G
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V

EPA 0523873 A1 discloses a modification of the compounds of EPA 0520823 A2 wherein Xaa^3 is phenylalanine or p-fluorophenylalanine.

EPA 0461869 describes compounds which inhibit farnesylation of Ras protein of the formula:(SEQ ID NO: 1)

Cys-Aaa^1-Aaa^2-Xaa

where Aaa^1 and Aaa^2 are aliphatic amino acids and Xaa is an amino acid. The aliphatic amino acids which are disclosed are Ala, Val, Leu and Ile. Preferred compounds are those where Aaa^1 is Val, Aaa^2 is Leu, Ile or Val and Xaa is Ser or Met. Preferred specific compounds are:

Cys-Val-Leu-Ser

Cys-Val-Ile-Met

Cys-Val-Val-Met

U.S. Pat. Nos. 5,141,851 and WO 91/16340 disclose the purified farnesyl protein transferase and certain peptide inhibitors therefor, including, for example, CVIM, TKCVIM and KKSKTKCVIM (SEQ ID NO:2 through SEQ ID NO:4),

WO 92/18465 discloses certain farnesyl compounds which inhibit the enzymatic methylation of proteins including ras proteins.

EPA 0456180 A1 is directed to a farnesylprotein transferase assay which can be used to identify substances that block farnesylation of ras oncogene gene products while EPA 0512865 A2 discloses certain cyclic compounds that are useful for lowering cholesterol and inhibiting farnesylprotein transferase.

As will be evident from the foregoing, there is a great deal of research effort directed towards the development of inhibitors of farnesyltransferase. However, there still remains a need for improvements in this critically important area.

SUMMARY OF THE INVENTION

An important embodiment of the present invention is based on the finding that a novel group of peptidomimetics as represented by Formula (I) have a high inhibitory potency against human tumor p21ras farnesyltransferase:

found to elute from the preferred affinity matrices disclosed in more detail hereinbelow.

While it is believed that advantages in accordance with the invention can be realized simply through affinity chromatography techniques, additional benefits will be achieved through the application of additional purification techniques, such as gel filtration techniques. For example, the inventors have discovered that Sephacryl S-200 high resolution gel columns can be employed with significant benefit in terms of protein purification. However, the present disclosure is by no means limited to the use of Sephacryl S-200, and it is believed that virtually any type of gel filtration arrangement can be employed with some degree of benefit. For example, one may wish to use techniques such as gel filtration, employing media such as Superose, Agarose, or even Sephadex.

Through the application of various of the foregoing approaches, the inventors have successfully achieved farnesyl transferase enzyme compositions of relatively high specific activity, measured in terms of ability to transfer farnesol from farnesyl pyrophosphate. For the purposes of the present invention, one unit of activity is defined as the amount of enzyme that transfers 1 pmol of farnesol from farnesyl pyrophosphate (FPP) into acid-precipitable $p21^{H\text{-}ras}$ per hour under the conditions set forth in the Examples. Thus, in preferred embodiments the present invention is concerned with compositions of farnesyl transferase which include a specific activity of between about 5 and about 10 units/mg of protein. In more preferred embodiments, the present invention is concerned with compositions which exhibit a farnesyl transferase specific activity of between about 500 and about 600,000 units/mg of protein. Thus, in terms of the unit definition set forth above, the inventors have been able to achieve compositions having a specific activity of up to about 600,000 units/mg using techniques disclosed herein.

Of principal importance to the present invention is the discovery that proteins or peptides which incorporate a farnesyl acceptor sequence, such as one of the farnesyl acceptor sequences discussed above, function as inhibitors of farnesyl:protein transferase, and therefore may serve as a basis for anticancer therapy. In particular, it has been found that farnesyl acceptor peptides can successfully function both as false substrates that serve to inhibit the farnesylation of natural substrates such as $p21^{ras}$, and as direct inhibitors which are not themselves farnesylated. Compounds falling into the latter category are particularly important in that these compounds are "pure" inhibitors that are not consumed by the inhibition reaction and can continue to function as inhibitors. Both types of compounds constitute an extremely important aspect of the invention in that they provide a means for blocking farnesylation of $p21^{ras}$ proteins, for example, in an affected cell system.

The farnesyl transferase inhibitor embodiments of the present invention concern in a broad sense a peptide or protein other than $p21^{ras}$ proteins, lamin a or lamin b, or yeast mating factor a, which peptide or protein includes a farnesyl acceptor sequence within its structure and is further capable of inhibiting the farnesylation of $p21^{ras}$ by farnesyl transferase.

In preferred embodiments, the farnesyl transferase inhibitor of the present invention will include a farnesyl acceptor or inhibitory amino acid sequence having the amino acids —C—A—A—X, wherein:

C=cysteine;

A=any aliphatic, aromatic or hydroxy amino acid; and

X=any amino acid.

Typically, the farnesyl acceptor or inhibitory amino acid sequence will be positioned at the carboxy terminus of the protein or peptide such that the cysteine residue is in the fourth position from the carboxy terminus.

In preferred embodiments, the inhibitor will be a relatively short peptide such as a peptide from about 4 to about 10 amino acids in length. To date, the most preferred inhibitor tested is a tetrapeptide which incorporates the —C1'A—A—X recognition structure. It is possible that even shorter peptides will ultimately be preferred for practice of the invention in that the shorter the peptide, the greater the uptake by such peptide by biological systems, and the reduced likelihood that such a peptide will be destroyed or otherwise rendered biologically ineffective prior to effecting inhibition. However, numerous suitable inhibitory peptides have been prepared and tested by the present inventors, and shown to inhibit enzymatic activities virtually completely, at reasonable concentrations, e.g., between about 1 and 3 μM (with 50% inhibitions on the order of 0.1 to 0.5 μM).

While, broadly speaking, it is believed that compounds exhibiting an IC_{50} of between about 0.01 μM and 10 μM will have some utility as farnesyl transferase inhibitors, the more preferred compounds will exhibit an IC_{50} of between 0.01 μM and 1 μM. The most preferred compounds will generally have an IC_{50} of between about 0.01 μM and 0.3 μM.

Exemplary peptides which have been prepared, t2ested and shown to inhibit farnesyl transferase at an IC_{50} of between 0.01 and 10 μM include CVIM; KKSKTKCVIM; TKCVIM; RASNRSCAIM; TQSPQNCSIM; CIIM; CVVM; CVLS; CVLM; CAIM; CSIM; CCVQ; CIIC; CIIS; CVIS; CVLS; CVIA; CVIL; CLIL; CLLL; CTVA; CVAM; CKIM; CLIM; CVLM; CFIM; CVFM; CVIF; CEIM; CGIM; CPIM; CVYM; CVTM; CVPM; CVSM; CVIF; CVIV; CVIP; CVII.

A variety of peptides have been synthesized and tested such that now the inventors can point out peptide sequencing having particularly high inhibitory activity, i.e., wherein relatively lower concentrations of the peptides will exhibit an equivalent inhibitory activity (IC_{50}) Interestingly, it has been found that slight changes in the sequence of the acceptor site can result in loss of inhibitory activity. Thus, when TKCVIM is changed to TKVCIM, the inhibitory activity of the peptide is reversed. Similarly, when a glycine is substituted for one of the aliphatic amino acids in CAAX, a decrease in inhibitory activity is observed. However, it is proposed that as long as the general formula as discussed above is observed, one will achieve a structure that is inhibitory to farnesyl transferase.

A particularly important discovery is the finding that the incorporation of an aromatic residue such as phenylalanine or tyrosine into the third position of the tetrapeptide will result in a "pure" inhibitor. As used herein, a "pure" farnesyl:protein transferase inhibitor is intended to refer to one which does not in itself act as a substrate for farnesylation by the enzyme. This is particularly important in that the inhibitor is not consumed by the inhibition process, leaving the inhibitor to continue its inhibitory function unabated. Exemplary compounds which have been tested and found to act as pure inhibitors include CVIF, CVFM, and CVYM. Pure inhibitors

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